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Nitrapyrin decreased nitrification of nitrogen released from soil organic matter but not *amoA* gene abundance at high soil temperature

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ABSTRACT

Water pulses have a significant impact on nitrogen (N) cycling, making management of N challenging in agricultural soils that are exposed to episodic rainfall. In hot, dry environments, wetting of dry soil during summer fallow causes a rapid flush of organic matter mineralisation and subsequent nitrification, which may lead to N loss via nitrous oxide emission and nitrate leaching. Here we examined the potential for the nitrification inhibitor nitrapyrin to decrease gross nitrification at elevated temperature in soils with contrasting soil organic matter contents, and the consequent effects on ammonia oxidiser populations. Soil was collected during summer fallow while dry (water content 0.01 g g^{-1} soil) from a research site with two management treatments (tilled soil and tilled soil with long-term additional crop residues) by three field replicates. The field dry soil (0–10 cm) was wet with or without nitrapyrin, and incubated (20 or 40 °C) at either constant soil water content or allowed to dry (to simulate summer drying after a rainfall event). Gross N transformation rates and inorganic N pools sizes were determined on six occasions during the 14 day incubation. Bacterial and archaeal *amoA* gene abundance was determined on days 0, 1, 7 and 14. Nitrapyrin increased ammonium retention and decreased gross nitrification rates even with soil drying at 40 °C. Nitrification was likely driven by bacterial ammonia oxidisers, as the archaeal *amoA* gene was below detection in the surface soil layer. Bacterial ammonia oxidiser gene abundances were not affected by nitrapyrin, despite the decrease in nitrifier activity. Increased soil organic matter from long-term additional crop residues diminished the effectiveness of nitrapyrin. The present study highlights the potential for nitrapyrin to decrease nitrification and the risk of N loss due to mineralisation of soil organic matter under summer fallow conditions.

1. Introduction

Regions where water-limited soils occur at high temperatures include those with semi-arid, arid and Mediterranean-type climates, which are widely used for agricultural production. Most agricultural mitigation strategies for nitrogen (N) loss are targeted towards increasing N fertiliser use efficiency, through for example, matching spatial and temporal N supply to crop N demand during the growing season (Meisinger and Delgado, 2002; Murphy et al., 2004). A large proportion of N losses in water-limited soils however can be in response to biochemical processes that occur during the dry, non-growing season (Mummey et al., 1997; Anderson et al., 1998; Austin et al., 2004; Barton et al., 2008). Management of these losses is challenging, as they occur in response to episodic rainfall events, rather than agricultural management practices. After summer rainfall in semi-arid environments, microorganisms rapidly become active, resulting in a flush of soil organic matter (OM) mineralisation that increases inorganic N availability (Murphy et al., 1998; Austin et al., 2004; Phillips et al., 2015). Production of inorganic N is particularly detrimental in fallow soil, as plant uptake is minimal and nitrate (NO_3^-) is at risk of loss by leaching during subsequent rainfall and drainage events (Anderson et al., 1998; Arregui and Quemada, 2006). In addition, up to half of annual emissions of the greenhouse gases nitric oxide (NO) and nitrous oxide (N_2O) can occur when hot, dry soil is wetted (Barton et al., 2008; Galbally et al., 2008; Barton et al., 2013b).

One strategy that decreases the potential for N loss is the use of a nitrification inhibitor. These chemicals control nitrification, the key pathway for N loss, often by binding or otherwise deactivating one of the enzymes involved (Slangen and Kerkhoff, 1984). Generally,

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the effectiveness of nitrification inhibitors decreases with increasing temperature, due to increasing microbial degradation, stimulation of microbial activity and loss of volatile chemicals (Slangen and Kerkhoff, 1984). Nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine) has been successfully used for many years to decrease nitrification and N loss from applied fertiliser where N inputs are high (45–338 kg N ha^{-1}; Wolt, 2004), and has been effective at soil temperatures as high as 25–35 °C (Ali et al., 2008; Chen et al., 2010). These studies have investigated the effectiveness of nitrapyrin in the presence of N fertiliser, however it is not clear if nitrapyrin inhibits nitrification due to N released from soil OM mineralisation.

The effectiveness of nitrapyrin at decreasing nitrification in soil depends on a number of interacting factors besides soil temperature (Slangen and Kerkhoff, 1984). Soil OM both absorbs nitrapyrin and provides an energy source for the microorganisms which degrade nitrapyrin, decreasing the ability of nitrapyrin to inhibit nitrification (Goring, 1962; Lewis and Stefanson, 1975). Semi-arid soils often have low OM contents due to the seasonally dry climate, low plant productivity and continual soil loss by erosion (Archibold, 1995; Ryan, 2011). We expected that nitrapyrin would more effectively inhibit nitrapyrin at elevated temperatures in a low OM soil compared to where additional crop residue inputs have increased soil OM. There is a paucity of research about the effect of soil wetting and drying events on the effectiveness of nitrapyrin, however some research suggests that nitrification inhibitors can be more effective when either low or high water content limits the activity of nitrifying organisms (Keeney, 1986). We therefore hypothesised that nitrapyrin would be more effective as soil dried and nitrification activity decreased.

The mechanism by which nitrapyrin inhibits nitrification is thought to involve ammonia monooxygenase (AMO), the main enzyme involved in ammonia oxidation (Vannelli and Hooper, 1992). Nitrapyrin is a substrate for AMO, producing 6-chloropicolinic acid which then binds indiscriminately to other membrane proteins, the suggested mechanism for inactivation of ammonia oxidation (Vannelli and Hooper, 1992). Nitrapyrin does not inhibit hydroxylamine oxidation or nitrite oxidation (the steps in nitrification following ammonia oxidation) except at extremely high concentrations (80–175 ppm; Campbell and Aleem, 1965a,b). The α subunit of AMO contains the enzyme's active site and is encoded by the *amoA* gene, which has homologous gene sequences in both ammonia-oxidising bacteria (AOB) and archaea (AOA; Nicol and Schleper, 2006). We therefore hypothesised that inhibition of AMO by nitrapyrin would diminish the ability of ammonia-oxidising microorganisms to obtain energy and to grow, thus decreasing *amoA* gene abundance. Few studies have examined the effect of nitrapyrin on ammonia oxidiser populations, and findings have been contradictory (Cui et al., 2013; Lehtovirta-Morley et al., 2013; Shen et al., 2013). Further research is necessary to unravel the many interacting factors that control the effectiveness of nitrapyrin at inhibiting ammonia-oxidising microorganisms and nitrification.

Consequently, we examined the potential of the nitrification inhibitor nitrapyrin to control nitrification at elevated soil temperature in response to a simulated rainfall wetting and drying event. Specifically, we determined (i) whether nitrapyrin decreased gross nitrification rates without altering other N transformation rates at 20 and 40 °C; (ii) whether increased soil OM content diminishes the ability of nitrapyrin to inhibit nitrification at elevated temperature; (iii) whether decreasing water content with time (as occurs when soil dries after a summer rainfall event) increases the ability of nitrapyrin to inhibit nitrification compared to when soil water content is optimal; and (iv) if populations of AOA or AOB are consequently affected.

2. Methods

2.1. Soil and soil collection

Soil was collected from the Liebe Group's Soil Biology Trial (30.00° S, 116.33° E), in the northern wheatbelt of Western Australia, approximately 221 km north–northeast of Perth. This research site was established in 2003 with a three year lupin–wheat–wheat rotation and a range of field management treatments to create a range of soil OM contents. Each treatment had three field replicate plots that are 80 m long and 10 m wide. Two treatments with contrasting OM contents were selected for the present study: tilled soil ('Tilled'), and tilled soil loaded with additional organic matter ('Tilled + OM'). The Tilled soil was tilled to 10 cm depth annually using offset discs before seeding, and seeded with knife point tines. Tilled + OM soil had 20 t ha^{-1} barley, canola, oat and oat chaff tilled into the soil in 2003, 2006, 2010 and 2012 respectively, using the same tillage method described for the Tilled soil. This represented an additional 36 t C ha^{-1}, of which 7.0 t C ha^{-1} was retained as extra soil organic carbon (SOC) in the Tilled + OM soil nine years after trial establishment (i.e. 64% more SOC in Tilled + OM than in Tilled soil; Table 1).

The region has a semi-arid climate, with hot, dry summers and cool, wet winters (when cropping occurs). Based on 15 years of climate data (1997–2014) the area has a mean annual rainfall of 284.9 mm, mean monthly temperatures ranging from 5.8 to 35.3 °C and actual temperatures ranging from −1.0 to 46.9 °C (Commonwealth of Australia Bureau of Meteorology, http://www.bom.gov.au/climate/data). At the research site, soil temperatures (5 cm depth) ranged from 6.2 to 45.6 °C (2008–2012). Soil at the site was a deep sand (92% sand, 2% silt, 6% clay) and classified as a Basic Regolithic Yellow-Orthic Tenosol (Australian soil classification; Isbell, 2002), or a Haplic Arenosol (World Reference Base for Soil Resources; IUSS Working Group WRB, 2007). Selected soil chemical and biological properties are listed in Table 1.

Soil was sampled in late summer (27 March 2012), prior to the first rains of the autumn/winter growing season. At this time, the soil was fallow and naturally air-dry (field soil water content of 0.01 g g^{-1} soil). Rain had last fallen in mid-summer (3 February 2012), 52 days prior to soil sampling, and over those 52 days, daily maximum soil temperatures at 5 cm depth ranged from 26 to 39 °C. A composite sample (40 cores, each 7 cm diameter by 10 cm depth) was taken from each replicate field plot in a zigzag sampling pattern. Samples were sieved (<2 mm) and stored without further drying at room temperature until further analysis.

Table 1
Properties of field soils (0–10 cm depth), collected nine years after soil organic matter (OM) treatments were imposed. Values are means ± standard error of the mean (SEM) ($n = 3$).

	Tilled soil	Tilled + OM soil
Soil pH_{CaCl_2} [b]	6.15 ± 0.18	6.23 ± 0.13
Total carbon (%)[c]	0.78 ± 0.03	1.31 ± 0.07[a]
Total carbon (t ha^{-1})	10.83 ± 0.55	17.78 ± 1.10[a]
Total nitrogen (%)[c]	0.06 ± 0.00	0.10 ± 0.01[a]
Soil C:N ratio	12.9 ± 0.2	12.9 ± 0.2
Ammonium-N (μg g^{-1})	2.25 ± 0.45	1.76 ± 0.57
Nitrate-N (μg g^{-1})	20.47 ± 1.05	31.83 ± 2.49
Bacterial *amoA* (gene copies g^{-1})	$2.08 \times 10^7 \pm 4.22 \times 10^6$	$2.01 \times 10^7 \pm 6.65 \times 10^6$
Archaeal *amoA* (gene copies g^{-1})	$<1 \times 10^3$	$<1 \times 10^3$

[a] Tilled + OM soil is significantly different from Tilled soil at $P < 0.01$.
[b] Soil pH measured in 0.01 M $CaCl_2$ with a 1:5 soil:extract ratio.
[c] Total C, and total N determined by dry combustion of finely ground soil using an Elementar vario MACRO CNS elemental analyser (Hanau, Germany).

2.2. Laboratory experimental design

The laboratory experimental design was two nitrification inhibitor treatments (with or without nitrapyrin), two incubation temperatures (20 and 40 °C), two soils of differing OM contents (Tilled and Tilled + OM) by three field replicates, and two soil water regimes (OWC and DRY; explained below). The nitrification inhibitor used was nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine) at 9 μg active ingredient g^{-1} of dry soil. The soil water regimes were: simulated rainfall event to optimal soil water content that was held at optimum over the course of the experiment (OWC); and a simulated rainfall event to optimal soil water content with subsequent drying (DRY). The optimal soil water content chosen was 45% water-filled pore space (WFPS), as (i) this is the WFPS that occurs following a common summer rainfall event for this region of 15 mm; (ii) measured field soil water content in this region rarely exceeds 45% WFPS (Barton et al., 2008, 2011, 2013b); and (iii) because at this WFPS neither mineralisation nor nitrification are constrained in this soil type (Gleeson et al., 2010).

2.3. Gross N transformation rates and inorganic N analysis

^{15}N isotopic pool dilution was used to calculate gross N cycling transformation rates. Paired treatments of ^{15}N were either ^{15}N enriched (60 atom%) ammonium sulphate [$(NH_4)_2SO_4$] + potassium nitrate (KNO_3) at natural abundance, or $(NH_4)_2SO_4$ at natural abundance + ^{15}N enriched (60 atom%) KNO_3. Both $(NH_4)_2SO_4$ and KNO_3 were applied at 5 μg N g^{-1}. In total, four solutions were prepared for application to the samples: $(^{15}NH_4)_2SO_4$ + KNO_3; $(NH_4)_2SO_4$ + $K^{15}NO_3$; $(^{15}NH_4)_2SO_4$ + KNO_3 + nitrapyrin; and $(NH_4)_2SO_4$ + $K^{15}NO_3$ + nitrapyrin. Each of the four ^{15}N solutions were then added to separate subsamples of soil, which were mixed well and then packed into 120 mL vials to 10 cm depth and to the bulk density at the research site (1.4 g cm^{-3} bulk density). OWC vials were placed inside sealed 500 mL glass jars with 5 mL of water at the bottom, to minimise evaporation. These jars were aerated every 24 h to prevent anaerobic conditions developing. DRY vials were incubated without lids. Samples were incubated at either 20 or 40 °C for up to 14 days.

Soils were extracted on six occasions during the incubation using individual subsamples for each time point: 2–4 h after ^{15}N addition, and at 1, 3, 7, 10 and 14 days. At each of these sampling times, soil was mixed and then a subsample (*ca.* 20 g) was snap-frozen in liquid nitrogen, and then stored at −80 °C for subsequent DNA analysis (see below). Another subsample of soil (*ca.* 30 g) was used to determine gravimetric soil water content. Water-filled pore space was calculated by dividing volumetric water content by total porosity, where volumetric water content is gravimetric water content multiplied by bulk density (1.4 g cm^{-3}), and total porosity is [1 − (bulk density/particle density)] × 100, using measured particle density for each field soil replicate (Linn and Doran, 1984).

A further subsample (20.0 g) was extracted with 80 mL of 0.5 M potassium sulphate (K_2SO_4) for 30 min in an end-over-end shaker, then filtered through Whatman No. 42 filter paper. The extracts were kept frozen at −20 °C until further analysis for inorganic N. Using Buchner funnels under vacuum, the inorganic N remaining in soil solution was removed by a second extraction with 80 mL of 0.5 M K_2SO_4 followed by two extractions with 80 mL of MilliQ water. The remaining washed soil was dried at 70 °C and ground to a fine powder, then analysed for ^{15}N atom% and total N using a continuous flow system, consisting of a SERCON 20–22 Stable Isotope Ratio Mass Spectrometer connected with an Automated Nitrogen Carbon analyser (Sercon, Crewe, UK).

Inorganic N concentrations of the extracts were determined by colorimetric analysis on a Skalar San Plus auto-analyser (Skalar Inc., Breda, The Netherlands), using the modified Berthelot reaction for NH_4^+-N (Krom, 1980) and the hydrazinium reduction method for NO_3^--N (Kamphake et al., 1967). The extracts from each sampling time were prepared for IRMS $^{15}N/^{14}N$ isotope ratio analysis using a modified diffusion method (Brooks et al., 1989; Sørensen and Jensen, 1991). The extract NH_4^+ and NO_3^- was trapped on separate acidified diffusion disc, and the discs were analysed by isotope ratio mass spectrometer as described above.

2.4. Calculation of gross N transformation rates

The analytical equations of Kirkham and Bartholomew (1954) were used to calculate gross N mineralisation and nitrification rates between each sampling time point (i.e. days 1–3, days 3–7, days 7–10, and days 10–14).

2.5. Nucleic acid extraction and quantitative PCR

DNA was extracted from 800 mg sub-samples of soil immediately after wet-up and from days 1, 7 and 14 of the incubation. The DNA PowerSoil® Kit (MoBio) was used following the manufacturer's instructions with one exception: the DNA was eluted in 50 μL of the final solution. DNA was stored at −40 °C prior to further analysis.

Bacterial and archaeal *amoA* genes were quantified by qPCR (Applied Biosystems ViiA™ 7) using GoTaq® qPCR System (Promega Corp.). For bacterial *amoA* gene quantification, primers used were amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) with fragment length of 491 bp (Rotthauwe et al., 1997). Each 20 μL qPCR reaction contained 10 μL of SYBR Green GoTaq® qPCR 2× Master Mix (Promega Corp.), 0.2 μL of each forward and reverse primer at a concentration of 10 μM, 2 μL bovine serum albumin (BSA, Ambion® UltraPure™ BSA, 5 mg/mL), 2 μL of template DNA and 5.6 μL of water. Cycling conditions were: 94 °C for 10 min, then 40 cycles of: 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and 78 °C for 30 s, followed by a melt curve. Fluorescence data was collected at the 78 °C stage.

For archaeal *amoA* gene quantification, primers used were Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) with fragment length of 635 bp (Francis et al., 2005). Each 10 μL qPCR reaction contained 5 μL of SYBR Green GoTaq® qPCR 2 × Master Mix (Promega Corp.), 0.1 μL of each forward and reverse primer at a concentration of 10 μM, 1 μL bovine serum albumin (BSA, Ambion® UltraPure™ BSA, 5 mg mL^{-1}), 1 μL of template DNA and 2.8 μL of water. Cycling conditions were: 94 °C for 10 min, then 40 cycles of: 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and 78 °C for 1 min, followed by a melt curve. Fluorescence data was collected at the 78 °C stage.

Each standard and sample was replicated three times during each qPCR run. Templates for determining gene copy numbers in the qPCR reactions were cloned plasmids as described in Barton et al. (2013a). The standard curves generated were linear over four orders of magnitude (10^3–10^6 gene copies) for AOB and over six orders of magnitude for AOA (10^3–10^8 gene copies) with r^2 values greater than 0.98. Amplification efficiencies ranged from 83 to 98% and a dilution series determined if there was any inhibition in the samples. The lower detection limit was 10^3 gene copies in 1 μL template.

2.6. Statistical analysis

Statistical differences between the field soil properties of the soil OM management treatments were examined using analysis of

variance (ANOVA) with associated TukeyHSD post hoc tests in R version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significances of nitrapyrin, soil OM management, temperature, and water regime treatments on WFPS, labelled NH_4^+ and NO_3^-, gross N transformation rates and *amoA* gene abundance with time were evaluated using a mixed model, PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Ammonia oxidiser *amoA* gene abundance data was $\log_{10}$ transformed before all analyses. Time was a significant factor for all variables, so each time point was analysed separately to better clarify statistical relationships between treatments (Table S1). R version 3.1.0 was used to run linear regressions of gross N transformation rates against ammonia oxidiser *amoA* gene abundance at the end of the period over which each rate was calculated.

3. Results

3.1. Recovery of ^{15}N

Two hours after $^{15}NH_4^+$ application, mean recovery of ^{15}N was 33% at 20 °C and 93% at 40 °C (Fig. S1a). Recovery of applied $^{15}NH_4^+$ at 20 °C increased to day 7, after which mean ^{15}N recovery was stable at 74%. The low recovery of ^{15}N two hours after $^{15}NH_4^+$ application at 20 °C was not due to nitrification, as there was no appearance of $^{15}NO_3^-$. Due to the low recovery of $^{15}NH_4^+$ at two hours, gross N mineralisation rates from 2 h to day 1 were not calculated. Mean recovery of applied $^{15}NO_3^-$ at both temperatures and all time points was approximately constant at 80% (Fig. S1b). In all treatments where $^{15}NO_3^-$ was applied, no remineralisation through to $^{15}NH_4^+$ was detected.

3.2. Water-filled pore space

Water-filled pore space in the OWC samples was maintained between 41 and 45% throughout the incubation (Fig. 1). Water-filled pore space in DRY samples decreased with time, from a mean of 43% to a minimum on day 14. Minimum mean WFPS in DRY samples was less in Tilled soil than in Tilled + OM soil [10 and 15% respectively at 20 °C (Fig. 1a); and 1 and 5% at 40 °C respectively (Fig. 1b); $P < 0.0001$].

3.3. Labelled ammonium and nitrate-N

After $^{15}NH_4^+$ application, nitrapyrin maintained labelled NH_4^+ pools approximately constant from day 3 at 40 °C, in contrast to labelled NH_4^+ pools without nitrapyrin which generally decreased with time, and were less than in the presence of nitrapyrin from day 3 until day 14 ($P < 0.0001$; data not shown). Nitrapyrin was more effective at retaining labelled NH_4^+ in soil without additional OM at 40 °C, and in soil held at OWC. Labelled NH_4^+ in Tilled + OM soil with nitrapyrin was less than in Tilled soil with nitrapyrin on all days of incubation ($P < 0.05$), and labelled NH_4^+ in DRY soil with nitrapyrin was less than in OWC soil with nitrapyrin from day 1 onwards ($P < 0.0001$). Labelled NH_4^+ at 20 °C followed the same general pattern as at 40 °C but on average was 27% of 40 °C labelled NH_4^+ (data not shown).

After $^{15}NH_4^+$ application, nitrapyrin kept labelled NO_3^- pools constant at 0.1 μg ^{15}N g^{-1} from days 1 to 14 at 40 °C, in contrast to labelled NO_3^- pools without nitrapyrin, which generally increased with time (Fig. 2c–d). There was no difference in labelled NO_3^- pools between Tilled and Tilled + OM soils or between OWC and DRY samples in the presence of nitrapyrin at 40 °C ($P < 0.05$; Fig. 2c–d). Without nitrapyrin however, labelled NO_3^- pools in Tilled soil were greater than in Tilled + OM soil from days 1–14 at 40 °C ($P < 0.0001$; Fig. 2c–d). Labelled NO_3^- at 20 °C generally followed a similar pattern and magnitude as at 40 °C (Fig. 2a–b).

3.4. Unlabelled inorganic N

Unlabelled NH_4^+ pools at 40 °C were greater in the presence of nitrapyrin than without from days 7–14 ($P < 0.0001$; Fig. 3c–d). Ammonium pools increased to a greater extent in the presence of nitrapyrin in Tilled + OM soil than in Tilled soil at 40 °C. Nitrapyrin retained more NH_4^+ in OWC samples than in DRY samples at 40 °C ($P < 0.001$). Nitrapyrin had a similar effect on NH_4^+ in soil incubated at 20 °C, but NH_4^+ pools were on average 40% of NH_4^+ pools at 40 °C (Fig. 3a–b).

Nitrapyrin kept unlabelled NO_3^- pools at 40 °C approximately stable in DRY soil, while NO_3^- pools decreased with time at OWC (Fig. 3g–h). Without nitrapyrin, NO_3^- pools generally increased with time to levels greater than in soil with nitrapyrin on days 10–14 at OWC ($P < 0.0001$) and on days 7–14 in DRY soil ($P < 0.01$). Tilled + OM soil had greater NO_3^- than Tilled soil in DRY samples from days 1 to 10 ($P < 0.05$), but at OWC, there was no difference in NO_3^- between OM treatments after 2 h ($P < 0.05$). Nitrate changes with time at 20 °C were more related to OM treatment than nitrapyrin (Fig. 3e–f). At OWC and 20 °C, NO_3^- pools in Tilled + OM soil were greater than Tilled soil until day 10, and nitrapyrin decreased the NO_3^- pool size in both OM treatments on days 10–14 ($P < 0.05$; Fig. 3g). Nitrate in DRY soil at 20 °C remained generally constant in all treatments from days 3 to 14 (Fig. 3h).

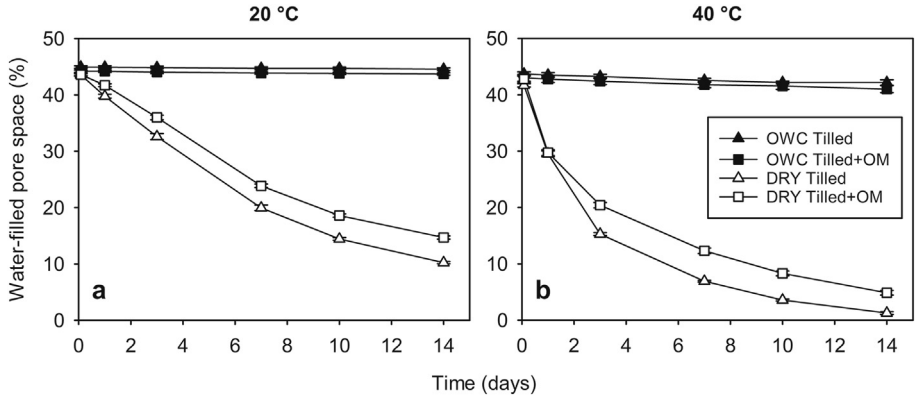

Fig. 1. Change in water-filled pore space (WFPS) through time (a) at 20 °C; and (b) at 40 °C. Error bars are ±SEM ($n = 12$) and are smaller than the symbols. Legend abbreviations: OWC, samples held at optimal water content (45% WFPS); DRY, samples wet-up to 45% WFPS then allowed to dry; OM, organic matter. Legend is the same for both panels.

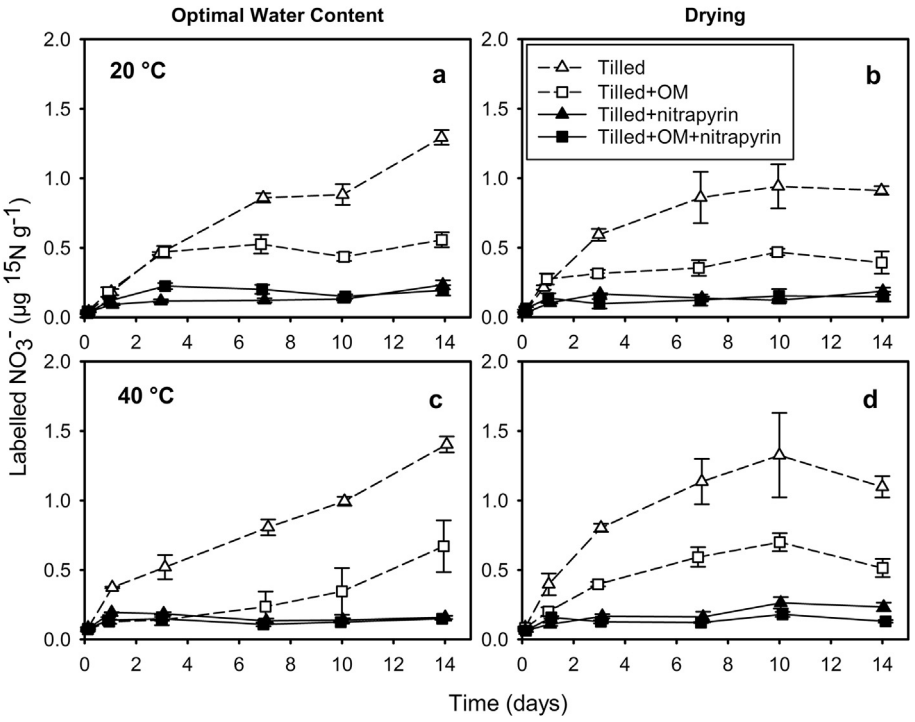

Fig. 2. Change in ^{15}N-labelled nitrate (NO_3^-) above natural abundance through time with added $^{15}(NH_4)_2SO_4$: (a) at 20 °C in soil held at optimal water content (45% WFPS); (b) at 20 °C in soil wet-up to 45% WFPS then allowed to dry; (c) at 40 °C in soil held at optimal water content; and (d) at 40 °C in soil wet-up then allowed to dry. Error bars are ±SEM ($n = 3$). Legend abbreviation: OM, organic matter. Legend is the same for all panels.

3.5. Gross N transformation rates

Nitrapyrin had no consistent effect on gross N mineralisation rates at either 20 or 40 °C ($P > 0.05$; Fig. 4a–d). Gross N mineralisation rates at 40 °C generally decreased with time, and means ranged from 0.1 to 7.1 μg N g^{-1} d^{-1} (Fig. 4c–d). Tilled + OM soil had greater gross N mineralisation rates than Tilled soil between 2 h and day 3 at 40 °C ($P < 0.05$). Optimal water content samples had greater gross N mineralisation than DRY samples between days 3 and14 at 40 °C ($P < 0.05$). In general, gross N mineralisation at 20 °C was half of gross N mineralisation at 40 °C, and followed a similar pattern over the incubation period (Fig. 4a–d).

There was no consistent effect of OM treatment, experimental water regime, or incubation temperature on gross nitrification rates (Fig. 4e–h). Mean gross nitrification rates at 40 °C ranged from 0 to 3.3 μg N g^{-1} d^{-1} (Fig. 4g–h). Addition of nitrapyrin decreased gross nitrification at 40 °C in DRY samples between days 1 and 7, and in OWC samples between days 1 and 3, and again between days 7 and 10 ($P < 0.05$). During these time periods at 40 °C, nitrapyrin inhibited nitrification by a mean of 86%. At 20 °C between days 1 and 3, nitrapyrin decreased gross nitrification in DRY Tilled soil and OWC Tilled + OM soil ($P < 0.05$; Fig. 4e–f). Furthermore, addition of nitrapyrin decreased gross nitrification at 20 °C in DRY samples between days 3 and 7 ($P < 0.0001$) and in OWC samples between days 7 and 14 ($P < 0.001$; Fig. 4e–f). For these samples at 20 °C, nitrapyrin inhibited nitrification by a mean of 62%.

3.6. Bacterial and archaeal amoA gene abundance

Archaeal *amoA* gene abundance was below detection limits in the majority of samples, and followed no pattern when detected (mean across all treatments: 3.30×10^4 gene copies g^{-1} dry soil; Fig. 5). Mean AOB gene abundance at 20 °C ranged from 4.32×10^5 to 7.28×10^7 gene copies g^{-1} dry soil (Fig. 5a–b). Wetting of dry soil at 20 °C decreased bacterial amoA gene abundance between time zero and day 1 in DRY samples ($P < 0.0001$) but not in OWC samples ($P > 0.05$; Fig. 5a–b). Addition of nitrapyrin at 20 °C decreased AOB gene abundance only on day 7, in the Tilled soil for both OWC and DRY water regimes, and in the DRY Tilled + OM soil ($P < 0.05$; Fig. 5a–b).

Mean bacterial *amoA* gene abundance at 40 °C ranged from below detection limits to 3.71×10^7 gene copies g^{-1} dry soil (Fig. 5c–d). Wetting of dry soil at 40 °C immediately decreased bacterial *amoA* gene abundance between time zero and day 1 in all 40 °C samples ($P < 0.0001$; Fig. 5c–d) but gene abundance recovered to similar levels as the original soil by day 14. Addition of nitrapyrin at 40 °C decreased AOB gene abundance only in the DRY Tilled soil on day 7 ($P < 0.05$; Fig. 5d). Otherwise there was no consistent effect of experimental water regime or OM treatment on AOB gene abundance at 20 or 40 °C.

Bacterial *amoA* gene abundance had no relationship with gross nitrification ($P > 0.05$), but had a statistically significant negative relationship with gross N mineralisation ($P < 0.05$; data not shown). The adjusted R-squared value for this relationship however was only 0.11, and the coefficient estimate was -0.052 ± 0.013 (standard error).

4. Discussion

Nitrapyrin has potential to decrease nitrification and thus the risk of N loss under elevated soil temperatures. Despite the fact that nitrapyrin is reported to become less effective at inhibiting nitrification with increasing temperature, the present study indicates that nitrapyrin was still able to inhibit nitrification at elevated temperatures in this semi-arid soil, without affecting other N transformation rates. Here we found that nitrapyrin had no effect on gross N mineralisation rates, indicating no inhibition of NH_4^+ supply for nitrification. However, gross nitrification rates were

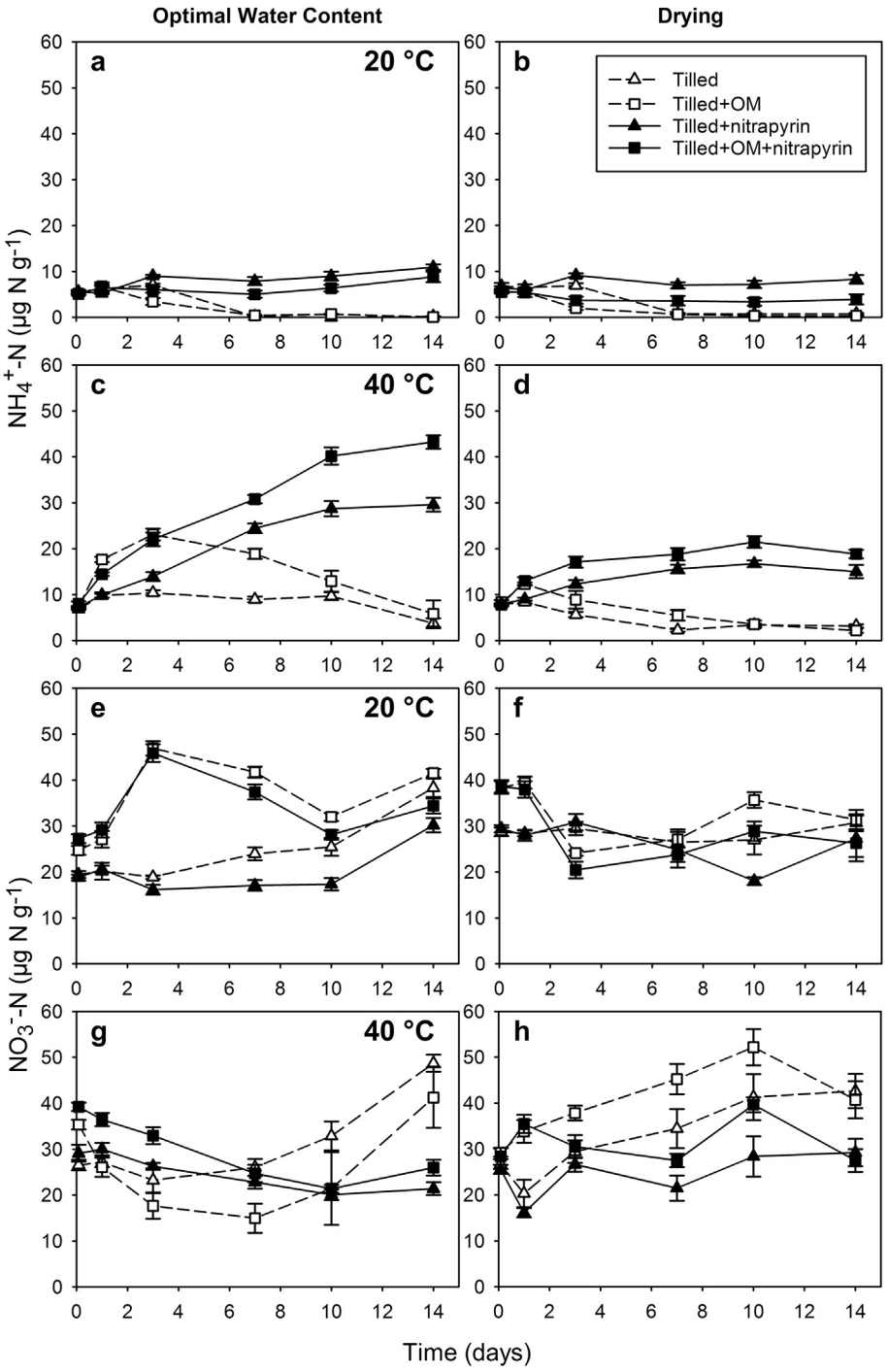

Fig. 3. Change in unlabelled inorganic N through time: (a) NH_4^+-N at 20 °C in soil held at optimal water content (45% WFPS); (b) NH_4^+-N at 20 °C in soil wet-up (to 45% WFPS) then allowed to dry; (c) NO_3^--N at 20 °C in soil held at optimal water content; (d) NO_3^--N at 20 °C in soil wet-up then allowed to dry; (e) NH_4^+-N at 40 °C in soil held at optimal water content; (f) NH_4^+-N at 40 °C in soil wet-up then allowed to dry; (g) NO_3^--N at 40 °C in soil held at optimal water content; and (h) NO_3^--N at 40 °C in soil wet-up then allowed to dry. Error bars are ±SEM ($n = 6$). Legend abbreviation: OM, organic matter. Legend is the same for all panels.

negligible in the presence of nitrapyrin, with this inhibition continuing at both 20 and 40 °C for the duration of the experiment. Other studies have found that nitrapyrin can decrease nitrification at temperatures from 25 to 35 °C (Ali et al., 2008; Chen et al., 2010). Most research to date has focussed on effectiveness of nitrapyrin at decreasing nitrification of NH_4^+-based fertilisers when applied during the cropping season (for example Chen et al., 1994; Wolt, 2004). When nitrapyrin is applied with a N source (such as N fertiliser), there is a more noticeable retention of applied inorganic N due to the higher NH_4^+ concentration (for example Tu, 1973). Our findings extend the use of nitrapyrin to control nitrification of soil OM mineralised outside the cropping season during summer fallow, with soil temperatures up to 40 °C.

Ammonia oxidiser gene abundance did not change in response to nitrapyrin, despite decreased gross nitrification rates and therefore ammonia oxidiser function. This is in contrast to our expectations that nitrapyrin would decrease ammonia oxidiser gene abundance, by diminishing energy production and potential

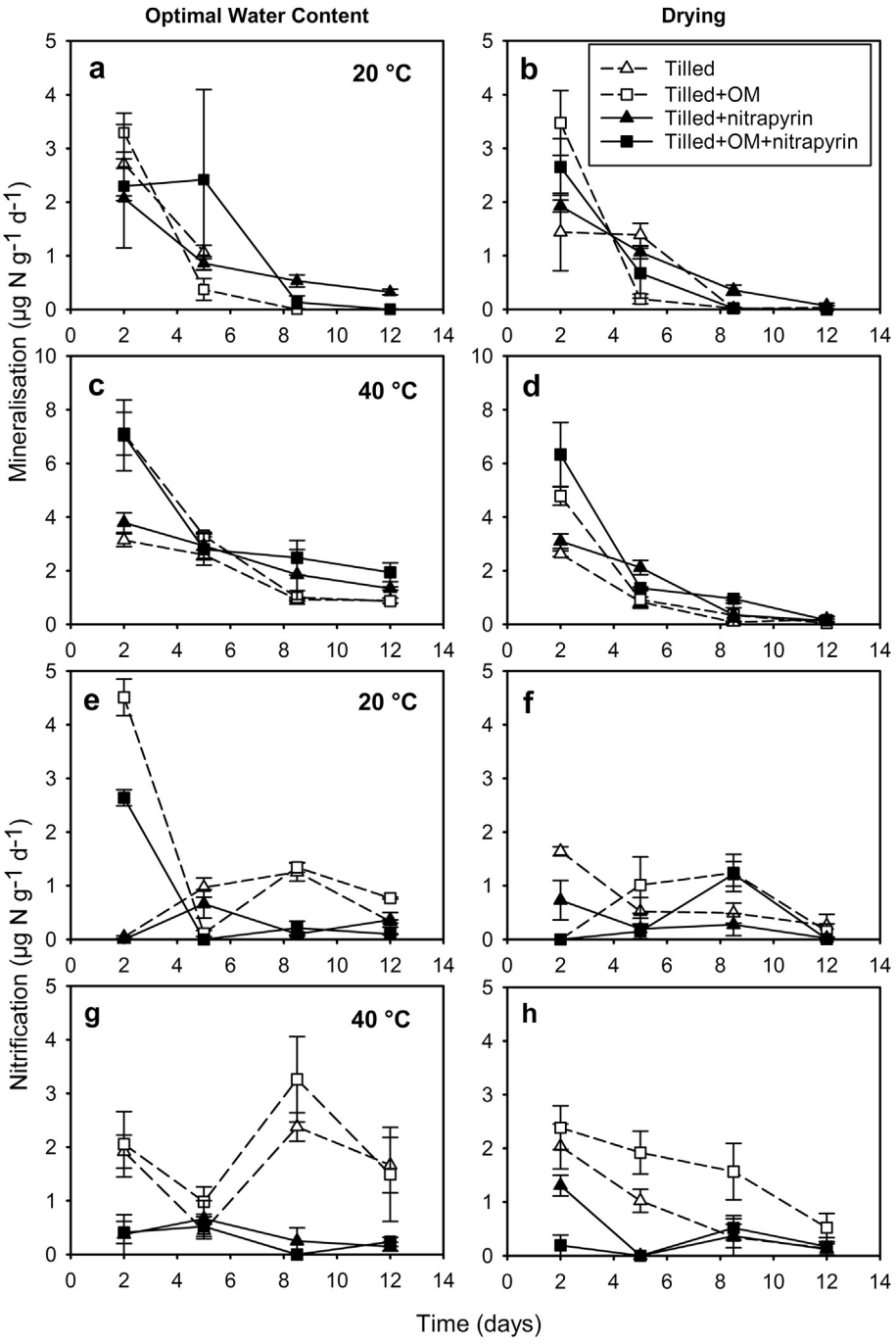

Fig. 4. Change in gross N mineralisation and nitrification rates through time. Gross N mineralisation (a) at 20 °C in soil held at optimal water content (45% WFPS); (b) at 20 °C in soil wet-up (to 45% WFPS) then allowed to dry; (c) at 40 °C in soil held at optimal water content; and (d) at 40 °C in soil wet-up then allowed to dry. Gross nitrification (e) at 20 °C in soil held at optimal water content; (f) at 20 °C in soil wet-up then allowed to dry; (g) at 40 °C in soil held at optimal water content; and (h) at 40 °C in soil wet-up then allowed to dry. Points shown are at the middle of the time period over which the rates were calculated (1–3 days; 3–7 days; 7–10 days; and 10–14 days). Error bars are ±SEM ($n = 3$). Legend abbreviation: OM: organic matter. Legend is the same for all panels. Note the different y-axis scales of (c) and (d).

for growth. Few studies have examined the effect of nitrapyrin on ammonia oxidiser gene abundance, and there is no clear evidence whether nitrapyrin affects AOA or AOB to a greater extent. Nitrapyrin decreased both growth and activity of the AOA *Nitrosotalea devanaterra* in liquid culture and soil (Lehtovirta-Morley et al., 2013), while nitrapyrin had weak inhibitory effects on nitrification and AOB but not AOA gene abundance in three Chinese soils (Cui et al., 2013). Nitrapyrin inhibited production of nitrite by the AOA *Ca*. Nitrososphaera viennensis but had only a weak inhibitory effect on production of nitrite by the AOB *Nitrosospira multiformis* in culture (Shen et al., 2013). Evidently, different strains and communities of ammonia oxidisers are influenced by nitrapyrin to differing degrees, likely also depending on environmental and experimental conditions. Here we attributed nitrification to AOB, as we were unable to detect AOA in the surface soil layer. For the same field trial Banning et al. (2015) also reported low AOA levels in the surface soil layer; although AOA gene abundance was of similar magnitude to AOB in deeper soil layers. They proposed the

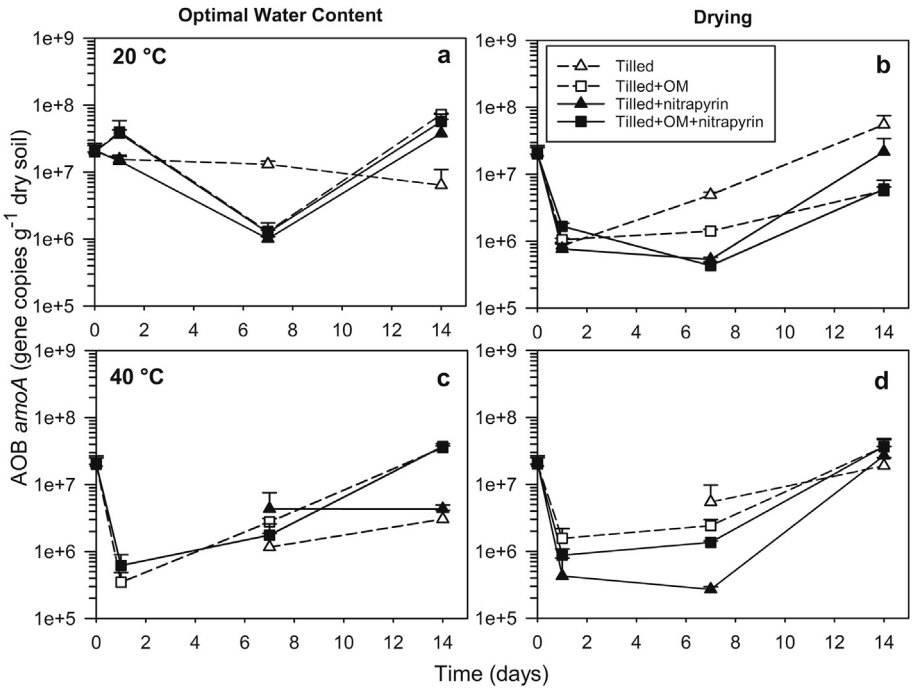

Fig. 5. Change in bacterial *amoA* gene abundance (AOB) through time (a) at 20 °C in soil held at optimal water content (45% WFPS); (b) at 20 °C in soil wet-up (to 45% WFPS) then allowed to dry; (c) at 40 °C in soil held at optimal water content; and (d) at 40 °C in soil wet-up then allowed to dry. Error bars are ±SEM ($n = 3$). Legend abbreviation: OM, organic matter. Legend is the same for both panels.

presence of archaea without nitrification capacity in the surface soil layer and illustrated a positive correlation between AOB gene abundance and gross nitrification in the soil profile (but a negative correlation between AOA and gross nitrification). Although AOB gene abundance was not affected by nitrapyrin, an effect on gross nitrification was still observed. Our results illustrate the need for further study to understand the complexities of ammonia oxidiser sensitivities to nitrapyrin.

Organic matter additions to this soil decreased the effectiveness of nitrapyrin, observed as a diminished retention of labelled NH_4^+. This was as expected, as nitrapyrin adsorbs onto OM, decreasing its ability to inhibit ammonia oxidation (Goring, 1962). Organic matter also increases soil microbial activity and provides carbon (C) and N substrates for microorganisms which degrade nitrapyrin (Goring, 1962). Recently there has been much interest in building soil OM particularly for the purpose of sequestering C to decrease atmospheric carbon dioxide levels and mitigate climate change (Powlson et al., 2011; Viscarra Rossel et al., 2014). Our results suggest that although nitrapyrin could be effective under summer conditions, these responses are likely to be greatest in low OM soils. Increasing soil OM, for example through crop residue additions as was done here, will have complex consequences on N cycling and our ability to manage N losses by the use of nitrapyrin.

Bacterial *amoA* gene abundance notably declined due to initial wet-up of dry soil, but was not affected by whether soil was subsequently held at optimal water content or allowed to dry. Rapid increases in soil water potential, as occur when rain falls on dry soil, place soil microorganisms under greater stress than they experience as soil dries (Schimel et al., 2007). If microorganisms are unable to adjust to the increasing water potential, they may release intracellular solutes, lyse and die (Kieft et al., 1987; Halverson et al., 2000). Recent evidence from *in situ* microbial communities suggests that soil microorganisms do not accumulate osmolytes as they dry (which might allow them to remain active; Boot et al., 2013), but instead the best strategy for survival is drought avoidance by dormancy until reactivation by a wetting event (Manzoni et al., 2014). The decline in AOB gene abundance after wet-up of dry soil, and the delay in recovery of AOB could also be due to some bacteria having decreased genome maintenance and repair during dormancy (Rittershaus et al., 2013). Instead, dormant bacteria may rely on repair systems that are more prone to errors and mutations, or induction of DNA repair systems upon reactivation (Rittershaus et al., 2013). Although we expected that microbial communities in this soil would be adapted to and able to cope with the climate (i.e. sporadic wetting events during the summer when soil is dry), a proportion of the AOB population appears not to be able to adjust rapidly enough to the increased water potential on soil rewetting. This is in contrast to the heterotrophic N mineralisers and immobilisers, which showed maximum activity during the first 24 h after wet-up. By day 14 however, bacterial *amoA* gene abundance in all treatments had recovered to the similar levels as in pre-wet soils. This follows a similar pattern to that observed in another semi-arid soil, where bacterial *amoA* gene abundance 72 h after wetting was the same or less than in pre-wet soil (Placella and Firestone, 2013).

Low recovery of $^{15}NH_4^+$ within two hours of application at 20 °C was attributed to rapid bacterial uptake. Uptake was followed by slow release of $^{15}NH_4^+$ back into the soil environment presumably once cells were saturated with N. This effect has been previously observed by Jones et al. (2013) using high-resolution nano-scale secondary ion mass spectrometry (NanoSIMS) stable isotope imaging: metabolically active bacterial cells in the rhizosphere of wheat plants accumulated and became saturated with $^{15}NH_4^+$ within 30 min of application of low levels of $^{15}NH_4^+$ (3 mM). In the present study we were not able to measure this bacterial $^{15}NH_4^+$ uptake due to the relatively enormous size of the organic N pool (307–1048 μg N g^{-1}) compared to the amount of applied ^{15}N (5 μg N g^{-1} at 60 atom%), and thus detected it as diminished ^{15}N recovery. Rapid bacterial uptake of applied $^{15}NH_4^+$ was not observed at 40 °C, which we attribute to limitation of microbial

immobilisation at elevated temperatures: in a similar semi-arid soil, Hoyle et al. (2006) noted that N immobilisation was restricted at temperatures greater than 30 °C, likely due to C substrate limitation. Our results imply that ^{15}N isotopic pool dilution may not be a useful tool to measure short-term rates (i.e. over the initial 24 h) of N transformations in N-limited soils, as these measurements appear to be confounded by rapid immediate bacterial uptake and release of $^{15}NH_4^+$ independent of soil OM mineralisation.

5. Conclusions

Managing N losses during summer fallow and following wetting events is challenging. The nitrification inhibitor nitrapyrin has the potential to retain mineralised NH_4^+ that is released after rainfall. However, nitrapyrin's effectiveness was decreased by increasing soil OM through long-term crop residue additions. Nitrapyrin lowered ammonia oxidiser activity but did not change *amoA* gene abundances. Instead, AOB populations were most affected by wet-up of dry soil, suggesting that these microbial communities are driven mainly by changes in environmental conditions rather than land management practices *per se*. Further research needs to evaluate whether nitrapyrin can be effective under field conditions, and whether this is an economic solution to potential N losses over summer fallow.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.05.029.

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